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Characterizing the behavioral effects of nerve agent-induced seizure activity in rats: Increased startle reactivity and perseverative behavior☆,☆☆

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ABSTRACT

The development and deployment of next-generation therapeutics to protect military and civilian personnel against chemical warfare nerve agent threats require the establishment and validation of animal models. The purpose of the present investigation was to characterize the behavioral consequences of soman (GD)-induced seizure activity using a series of behavioral assessments. Male Sprague–Dawley rats (n = 24), implanted with a transmitter for telemetric recording of encephalographic signals, were administered either saline or 1.0 LD_{50} GD (110 μg/kg, sc) followed by treatment with a combination of atropine sulfate (2 mg/kg, im) and the oxime HI-6 (93.6 mg/kg, im) at 1 min post-exposure. Seizure activity was allowed to continue for 30 min before administration of the anticonvulsant diazepam (10 mg/kg, sc). The animals that received GD and experienced seizure activity had elevated startle responses to both 100- and 120-dB startle stimuli compared to control animals. The GD-exposed animals that had seizure activity also exhibited diminished prepulse inhibition in response to 120-dB startle stimuli, indicating altered sensorimotor gating. The animals were subsequently evaluated for the acquisition of lever pressing using an autoshaping procedure. Animals that experienced seizure activity engaged in more goal-directed (i.e., head entries into the food trough) behavior than did control animals. There were, however, no differences between groups in the number of lever presses made during 15 sessions of autoshaping. Finally, the animals were evaluated for the development of fixed-ratio (FR) schedule performance. Animals that experienced GD-induced seizure activity engaged in perseverative food trough-directed behaviors. There were few differences between groups on other measures of FR schedule-controlled behavior. It is concluded that the GD-induced seizure activity increased startle reactivity and engendered perseverative responding and that these measures are useful for assessing the long-term effects of GD exposure in rats.

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1. Introduction

Soman (GD; pinacolyl methyl phosphonofluoridate) is a highly toxic organophosphorus (OP) compound that was originally developed as a chemical warfare nerve agent and still represents a major threat to both military and civilian personnel. The toxic effects of GD are primarily due to the irreversible inhibition of the enzyme acetylcholinesterase (AChE), resulting in the accumulation of acetylcholine (ACh) at the synapse and neuromuscular junction and over-stimulation of the cholinergic system. GD inhibition of AChE occurs through the binding of GD at the active serine site of AChE. Once bound, this complex rapidly undergoes dealkylation ("aging"), resulting in a stable monoalkylphosphonylated complex with AChE, and resumption of normal AChE activity requires de novo synthesis (reviewed in Marrs et al., 2006). The central nervous system (CNS) effects of nerve agents in humans include giddiness, anxiety, restlessness, headache, tremor, confusion, failure to concentrate, convulsions, respiratory depression, and respiratory arrest (Marrs, 2007).

The rapid inhibition of AChE and subsequent increase in synaptic ACh levels can lead to the development of seizure activity that can rapidly progress to *status epilepticus* (de Araujo Furtado et al., 2010; McDonough and Shih, 1997; McDonough et al., 2009). If the seizure activity is left untreated, profound brain damage can occur (Baille et al., 2005; McDonough and Shih, 1997; Shih et al., 2003). In fact, McDonough et al. (1995) showed that at least 20 min of seizure activity is necessary for neuropathological damage to occur in rats following nerve agent exposure. Nerve agent-induced seizures produce the most pronounced neuropathology in the piriform cortex, thalamus, amygdala,

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☆☆ The experimental protocol was approved by the Animal Care and Use Committee at the United States Army Medical Research Institute of Chemical Defense, and all procedures were conducted in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals* and the Animal Welfare Act of 1966 (P.L. 89-544), as amended.

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and hippocampus (Apland et al., 2010; Baille et al., 2001, 2005; Collombet et al., 2005; Filliat et al., 1999; Kadar et al., 1995; Lemercier et al., 1983; McDonough et al., 1986, 1998; McLeod, 1985; Modrow and Jaax, 1989; Petras, 1981, 1994; Raveh et al., 2002, 2003; Shih et al., 2003; Tryphonas and Clement, 1995) and contribute to long-term behavioral and cognitive deficits (Brandeis et al., 1993; Buccafusco et al., 1990; Collombet et al., 2008; Coubard et al., 2008; Filliat et al., 2007; Raffaele et al., 1987; Raveh et al., 2002, 2003).

There are numerous reports of behavioral deficits resulting from seizure-inducing levels of GD exposure. McDonough et al. (1986) reported a significant negative correlation between the severity of GD-induced neuropathology and the rate of acquisition of DRL (differential reinforcement of low rate responding) schedule performance. Haggerty et al. (1986) examined the acoustic startle response (ASR) of rats in response to GD challenge and reported decreased startle magnitude at 2 h following exposure to 0.8 LD_{50} (150 μg/kg, im) GD; however, they did not assess the startle response at later time points. In contrast, Philippens et al. (2000, 2005) reported elevated ASRs in guinea pigs at 2 and 24 h following 2.0 LD_{50} (49 μg/kg, sc) GD exposure. Joosen et al. (2009) reported mnemonic impairments in the Morris water maze at 8 weeks following 1.8 LD_{50} (200 μg/kg, sc) GD exposure in rats. Coubard et al. (2008) observed anxiety-like behaviors in mice at 30 and 90 days following 1.2 LD_{50} (110 μg/kg, sc) GD exposure. Auditory and contextual fear conditioned responses were also increased in these mice at 30 days post-exposure. On the other hand, Moffett et al. (2011) observed a severe impairment in auditory and contextual fear conditioning at approximately 1 week following 1.0–1.2 LD_{50} (110–132 μg/kg, sc) GD exposure in rats. Differences in species, time span, and neuropathology may account for the discrepancies between some of these reports of GD-induced behavioral deficits.

The purpose of the present study was to investigate the effects of GD-induced seizure activity on a series of behavioral tests (see Table 1). Three different behavioral procedures were chosen for inclusion in this experiment. First, ASR and reflex modification techniques (prepulse inhibition, PPI) (Davis, 1984) were chosen because these procedures have been used in both the rat (Haggerty et al., 1986) and guinea pig (Philippens et al., 2000, 2005) models of GD exposure, and lesions of the basolateral amygdala (Wan and Swerdlow, 1997) and the entorhinal cortex (Goto et al., 2002) have been shown to reduce PPI in rats without changing startle amplitude. However, these unconditioned behaviors have not been systematically evaluated in animals exposed to seizure-inducing levels of GD. Second, we chose to investigate the acquisition of lever-pressing using an autoshaping procedure (Sparber, 2001). Lesion studies have demonstrated that limbic structures typically damaged by GD-induced seizures (i.e., hippocampus) are necessary for the development of autoshaped responding in multiple species (Good and Honey, 1991; Hall et al., 1996; Reilly and Good, 1989; Richmond and Colombo, 2002). Furthermore, these procedures have been used extensively to detect the effects of neurotoxic compounds (Cohen et al., 1987; Fossom et al., 1985; Messing et al., 1988). Finally, we chose to evaluate the development of fixed-ratio (FR) schedule performance and the animals' abilities to adapt to changing reinforcement requirements. These techniques have been shown to be sensitive to the effects of a wide range of neurotoxic chemicals (Cory-Slechta, 1986; Gentry and Middaugh, 1988; Gerbec et al., 1988; Hojo et al., 2002; Middaugh and Gentry, 1992; Newland et al., 1986, 1994; Paletz et al., 2006), and Rabe and Haddad (1968) showed that hippocampal lesions in rats increased responding under an FR 20 schedule. The results of these experiments will be used to characterize nerve agent-induced seizure-related behavioral deficits.

Table 1
Sequence of phases, conditions, number of sessions, and the post-exposure day of testing.

Phase	Condition	Schedule	Number of sessions	Post-exposure day[a]
ASR	Baseline (pre-exposure)		3	
	Post-exposure		3	7–9
Food restriction				15–16
Operant acquisition	Magazine training		2	17–18
	Autoshaping		15	22–42
	Lever press training		4	43–46
	Reinforcement equalization		5	47–53
	Fixed ratio	FR 1	3	56–58
		FR 5	3	59–64
		FR 25	3	65–67
		FR 75	3	70–72
		FR 5	3	73–77

[a] Post-exposure days were counted from the first day of exposure.

2. Methods

2.1. Subjects

Twenty-four adult male Sprague–Dawley rats (pre-exposure weights: mean 475 g, range 422–563 g) were obtained from Charles River Laboratories (Kingston, NY, USA). Upon arrival, they were acclimated for 5 days and observed for evidence of good health. Animals were housed individually in polycarbonate cages in a temperature (21 ± 2 °C) and humidity (50 ± 10%) controlled colony room maintained on a reversed 12-h light–dark cycle with lights off at 0900 h. All experimental manipulations were conducted during the dark phase of the light–dark cycle when the animals are the most active. Food and water were available ad libitum in home cages. Animals were allowed to acclimate to the colony room (>1 week) before experimental procedures began. One week prior to the autoshaping phase (see Table 1 and below), the animals were placed under caloric regulation. This consisted of allotting the animals an amount of food equal to 90% of their estimated daily energy requirements (112 kcal/body weight $^{0.75}$) (Subcommittee on Laboratory Animal Nutrition, 1995). When applicable, the animals were fed at least 1 h following testing sessions. Water was available ad libitum in the home cage.

2.2. Surgery

2.2.1. Transmitter implantation

Approximately 1 week before experimentation, 16 animals were implanted with transmitters (F40-EET; Data Science International, St. Paul, MN, USA) to record electroencephalographic (EEG) activity and body temperature. The animals were anesthetized with isoflurane (3% induction; 1.5–2% maintenance with oxygen) and placed in a stereotaxic apparatus. One pair of cortical screws was placed bilaterally 2 mm from midline and 4 mm caudal to bregma. A second pair was placed 2 mm from midline and 1.5 mm rostral relative to lambda. The transmitters were implanted midscapular (sc), and the electrodes passed sc and wrapped around the cortical screws before being encased in dental acrylic. The incisions were sutured and treated with topical antibiotic ointment. For additional methods on transmitter implantation, see Williams et al. (2006). Animals were removed from the stereotaxic apparatus, placed on a circulating hot water blanket until consciousness was regained, and given buprenorphine (0.05 mg/kg, sc) before being returned to the colony room. Since there were a limited number of transmitters, the remaining 8 animals underwent sham surgeries. The sham surgeries were identical to the transmitter implantation surgeries with the exception that no transmitter was implanted. All animals were allowed 1 week to recover before further experimental manipulations were performed.

2.2.2. Transmitter removal

Since the transmitters were primarily used to differentiate animals that had seizures in response to GD-exposure from those that did not seize, the transmitters were removed under isoflurane anesthesia the day after ASR testing was completed (see Table 1). A small incision was made at the base of the skull, and the electrode wires were cut. A second incision was made in the midscapular region to remove the transmitter. The incisions were sutured and treated with topical antibiotic ointment. The animals were placed on a hot water blanket, given buprenorphine HCl (0.05 mg/kg, sc), and allowed to remain there until they had regained consciousness before being returned to the colony room. The animals were allowed 5 days to recover before further experimental manipulations were conducted. Sham removal surgeries were conducted in a similar manner, with the exception that there were no transmitters to remove.

2.3. Chemicals

Saline (0.9% NaCl) and sterile water, United States Pharmacopeia (USP), for injection were purchased from Hospira Inc. (Lake Forest, IL, USA). Atropine sulfate was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). HI-6 dimethanesulfonate salt was prepared by Starkes Associates (Buffalo, NY, USA) under contract to the Walter Reed Army Institute of Research (Silver Spring, MD, USA). Attane™ (isoflurane, USP) was purchased from Minrad Inc. (Bethleham, PA, USA). Buprenorphine HCl was purchased from Reckitt Benckiser Pharmaceuticals Inc. (Richmond, VA, USA). Topical antibiotic (bacitracin) was purchased from Perrigo (Allegan, MI, USA). Diazepam (USP), which was compounded with 40% propylene glycol, 10% ethanol, 5% sodium benzoate and benzoic acid, and 1.5% benzyl alcohol, was purchased from Hospira Inc. GD (pinacolyl methylphosphonofluoridate) was obtained from the US Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD, USA). Chemicals used in transcardial perfusion (4% paraformaldehyde, saline in phosphate buffer) as well as the 20% sucrose in phosphate buffer, were purchased from FD Neurotechnologies (Catonsville, MD, USA).

GD was diluted in sterile saline to a concentration of 220 μg/ml and administered sc at a volume of 0.5 ml/kg. Atropine sulfate (4 mg/ml) and HI-6 (187.2 mg/ml) were prepared in sterile water and administered intramuscularly (im) at a volume of 0.5 ml/kg. Diazepam (5 mg/ml) was administered sc at a volume of 2.0 ml/kg.

2.4. Apparatus

2.4.1. Acoustic startle response

ASR was measured in eight commercially purchased startle response chambers (Hamilton Kinder, Poway, CA, USA). Each sound-attenuated chamber was equipped with a piezoelectric accelerometer attached to a Plexiglas base for the transduction of animal movements (calibrated daily for accuracy). During testing sessions, the animal's movements were minimized by its placement in clear Plexiglas containers (8.9 × 17.8 cm with an adjustable ceiling set to 8.0 cm). Auditory stimuli were presented through a loudspeaker mounted 24 cm above the animal. A modified Realistic sound level meter (Hamilton Kinder, Poway, CA, USA) with the microphone placed in the location of the subject's head was used to calibrate the sound pressure level (SPL).

2.4.2. Operant testing apparatus

Operant testing was conducted in eight commercially available operant conditioning chambers (Med-Associates, Georgia, VT, USA). Each chamber was enclosed in a ventilated, light- and sound-attenuating cubicle and equipped with two retractable response levers (requiring approximately 0.22 N to operate), an opening centered between the levers through which 45-mg food pellets (Bio-Serv, Frenchtown, NJ, USA, Product #F0165) could be delivered, and a cue light above each lever. Following the completion of autoshaping (see Table 1), the left retractable lever was removed and a fixed lever was installed in its place. The food trough contained an infrared emitter-detector pair for monitoring entries. Illumination of the chamber was accomplished via a house light mounted on the wall opposite the response levers. White noise and tones were generated from a speaker located beneath the house light. Reinforcement contingencies and data collection were accomplished with 10 ms resolution using a computer running MED-PC IV® software (Med-Associates, Georgia, VT, USA).

2.5. Pharmacological procedures

On the day of exposure, animals were removed from the colony room and transported to a laboratory for exposure. Each animal received either 110 μg/kg GD (n = 16) or saline (n = 8) sc, and 1 min later 2 mg/kg atropine sulfate and 93.6 mg/kg HI-6 were administered im to increase post-exposure survivability. The LD_{50} value for GD given sc in rats is 110 μg/kg (Shih, 1990). The animals were promptly returned to the colony room where EEG activity (n = 12, GD; n = 4, control) was monitored for evidence of seizure activity. Body temperature and physical activity were also monitored via the transmitter, whereas signs of cholinergic crisis were monitored by visual observation. Thirty min following the appearance of electrographic seizure activity (i.e., rhythmic high-amplitude spikes that lasted at least 10 s (D'Ambrosio et al., 2009; de Araujo Furtado et al., 2009)), 10 mg/kg diazepam was administered sc to attenuate convulsions and standardize seizure duration. Sham-operated animals (n = 4, GD; n = 4, control) were visually monitored for signs of behavioral seizures and administered 10 mg/kg diazepam 30 min following the onset of Stage 3 behavioral seizures according to the Racine scale (Racine, 1972) (Stage 1, immobility; Stage 2, forelimb and/or tail extension, rigid posture; Stage 3, repetitive movements, head bobbing; Stage 4, rearing and falling; Stage 5, continuous rearing and falling; Stage 6, severe tonic–clonic seizures). In the absence of electrographic seizure activity or behavioral seizures below Stage 3 on the Racine scale, 10 mg/kg diazepam was administered sc at 120 min post-exposure.

2.6. Behavioral procedures

2.6.1. Acoustic startle response

Subjects were individually placed into an acoustic startle chamber and allowed to acclimate to the apparatus one session per day for three days prior to GD exposures. These sessions constituted the pre-exposure baseline. Each session began with a 3-min adaptation period with an ambient noise level of 60-dB SPL (full spectrum, 2–40 kHz). Following the adaptation period, 10 each of six unique trials were presented in randomized blocks; trials were separated by a 15 ± 5 s inter-trial interval (ITI). Six trial types were employed: 120-dB noise bursts alone or with prepulse, 100-dB noise bursts alone or with prepulse, 70-dB prepulse-only trials and no stimulus (60-dB ambient noise). Prepulse trials consisted of a 20-ms burst of 70-dB white noise presented 100 ms before a 40-ms burst of the startle eliciting stimulus (100- or 120-dB white noise). Pulse-only trials consisted of a 40-ms (1–2 ms rise/fall time) burst of white noise (60-, 70-, 100-, and 120-dB). The 60- and 70-dB stimuli were stimulus control conditions presented to ensure that there was not significant activity within the recording chamber during testing and to ensure that the 70-dB stimulus alone did not elicit a startle reflex. Each animal's movement was measured for a period of 200 ms following the onset of the test stimulus. The peak startle amplitude (V_{max}) was recorded as the highest observed force occurring during the 200-ms measurement window. The latency to peak startle amplitude (T_{max}) was the time that V_{max} occurred following the test stimulus onset. The amount of PPI produced was calculated following behavioral testing and equaled the difference in startle magnitude between the pulse-alone and the prepulse plus pulse trials, divided by the startle magnitude for the pulse-alone trials, multiplied by 100. A total of 6 ASR testing sessions

were conducted, 3 prior to and 3 following GD exposure (post-exposure days 7–9). Table 1 shows the order of behavioral testing across the entire experiment, including the number of sessions for each condition and the days post-exposure the testing occurred.

2.6.2. Autoshaping

Following the completion of acoustic startle testing, all animals were allowed 5 days to regain any weight lost as a consequence of GD exposure before being placed under controlled-feeding as described above for two calendar days before commencing magazine training. Subjects were exposed to a variable-time (VT) 60-s schedule of food presentation for two consecutive days before the autoshaping procedures were introduced. Magazine training sessions lasted approximately 20 min. During each session, the house light was illuminated and white noise present (75 dB). Each food pellet delivery was accompanied by a 400-Hz tone (500-ms duration). The retractable response levers remained retracted and cue lights extinguished throughout the duration of magazine training. Subjects were subsequently exposed to an autoshaping procedure (as described in Bushnell, 1988; Sparber, 2001) with the addition of a 9-s delay of reinforcement. Briefly, each session consisted of 32 trials separated by a variable ITI averaging 35 s (range 15 to 55 s). Each trial began with the presentation of the conditioned stimulus (CS; the insertion of the left retractable lever and simultaneous illumination of the left cue light). If the animal depressed the lever with sufficient force to register a response within 15 s of its insertion, a food pellet was delivered following a 9-s delay. If the animal failed to register a lever press, the left lever was retracted, the left cue light extinguished, a single food pellet was delivered following a 9-s delay, and the next ITI initiated. Sessions lasted approximately 45 min. Subjects were run under these conditions for a total of 15 sessions. If, after the 15th session, an animal had made less than 10 lever presses in any single session, additional sessions were conducted during which the animal was trained to lever press by the method of "successive approximations" (i.e., hand-shaping) with immediate delivery of reinforcement. Once all animals were reliably making lever press responses, additional sessions were conducted under the discrete trials procedure with a FR 1 in effect to equate the reinforcement history of all animals before initiating the fixed-ratio transitions (see Table 1).

2.6.3. Fixed-ratio (FR) transitions

After all animals had acquired lever pressing and their reinforcement histories had been equated, a series of increasing fixed-ratio (FR) schedules was introduced in which a fixed number of lever presses were required for each reinforcer delivery. Each session lasted for 60 min or 100 reinforcer deliveries, whichever occurred first, and began with the onset of the house light, 75-dB white noise, and illumination of the left cue light. A FR 1 schedule was in effect initially; one lever press was required for each reinforcer delivery. Thereafter, the requirements to obtain each reinforcer delivery were systematically increased by imposing schedule values of FR 5, FR 25, FR 75, and FR 5 with each FR value in effect for 3 consecutive sessions as described in Paletz et al. (2006).

2.7. Neuropathology assessments

At study completion, animals were deeply anesthetized, euthanized by exsanguination and transcardially perfused with 0.9% saline in 0.1 M phosphate buffer followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were post-fixed in paraformaldehyde for 6 h and then transferred to a 20% sucrose solution and frozen until sectioned. Brains were sectioned coronally (50 μm) and silver-stained by FD Neurotechnologies using the FD Neurosilver Kit I to determine the neuropathological effects of GD exposure. One series of sections was stained with cresyl violet to identify the brain regions of interest (i.e., amygdala, fiber tracts, hippocampus, piriform cortex, and thalamus) using *The Rat Brain in Stereotaxic Coordinates* (Paxinos and Watson, 2005) as a reference, and another series was stained with silver nitrate to detect degenerating fibers in these regions. Brain damage was qualitatively scored by trained technicians blinded to the experimental groups through evaluation of the silver-stained slices. Each area of injury was assigned a score from 0 (no damage/baseline stain intensity) to 4 (severe injury with entire given region darkly stained) based on the approximate percentage of tissue involvement as described previously (McDonough et al., 1995): 0 — no lesion; 1 — minimal, 1–10%; 2 — mild, 11–25%; 3 — moderate, 26–45%, 4 — severe, >45%.

2.8. Statistical analysis

Two GD-exposed rats were humanely euthanized before the completion of behavioral testing, and their data was omitted from all analyses except for latency to seizure induction and seizure duration. One animal was euthanized the day following GD exposure due to the presence of blood in the urine, which we have previously observed to occur in a small number of animals exposed to agent (less than 5%; unpublished data). The second was euthanized 14 days after exposure due to a failure to maintain body weight. The remaining GD-exposed animals were classified according to the occurrence of seizure activity on the day of GD exposures. Therefore, there are three experimental groups: controls (n = 8), 1.0 LD_{50} GD no seizure (n = 6), and 1.0 LD_{50} GD seizure (n = 8).

Statistical analyses were conducted using SPSS® 17.0 (SPSS Science, Chicago, IL, USA). For each dependent variable, a repeated measures analysis of variance (ANOVA) was conducted. If, for a dependent variable, there were violations of the assumption of homogeneity of variance, a logarithmic (base 10) or arcsine transformation was conducted prior to the ANOVA. Unless otherwise indicated, transformations successfully compensated for the heterogeneity of variance. For all analyses, a Huynh–Feldt's procedure was used to adjust for violations of assumptions of sphericity of repeated measures and adjusted p values are reported. Main effects of within-subject factors were evaluated using Bonferroni's procedure. Main effects of group were evaluated with Dunnett's procedure. Significant interactions were followed by tests of simple main effects. For all analyses, $\alpha = 0.05$.

3. Results

Ten out of the 16 animals exposed to GD experienced electrographic seizures or had Stage 3 behavioral seizures, and only these rats showed neuropathological damage in the brain regions of interest. The average (± standard error of the mean (SEM)) latency to seizure induction was 10.25 ± 3.22 min, and the average seizure duration was 193.4 ± 5.8 min. Fig. 1 shows body temperature as a function of time relative to GD exposure. The results of the two-way repeated measures ANOVA on body temperature revealed a significant main effect of group [F (2, 11) = 4.27, $p = 0.042$], a significant main effect of hour [F (36, 396) = 6.3, $p < 0.001$], and a significant interaction between group and hour [F (72, 396) = 2.43, $p = 0.021$]. A repeated ANOVA revealed that GD-exposed rats that did not display seizures had significantly decreased body temperature from 1 to 27 h after GD exposure relative to their baseline body temperature [F (36, 108) = 22.35, $p < 0.001$]. GD-exposed rats that displayed seizures had significantly decreased body temperature from 5 to 24 h after GD exposure relative to their baseline [F (36, 216) = 3.47, $p < 0.001$]. Saline control rats did not have a significant decrease in body temperature in the hours after exposure.

3.1. Body weight recovery

For statistical analyses, body weights (percent control) were averaged across calendar weeks before the ANOVA (data not shown), which yielded a significant main effect of group [F (2, 21) = 5.44, $p = 0.012$], a significant main effect of week [F (3, 61) = 6.75, $p = 0.001$] and a significant interaction between group and week [F (6, 61) = 3.51, $p = 0.005$].

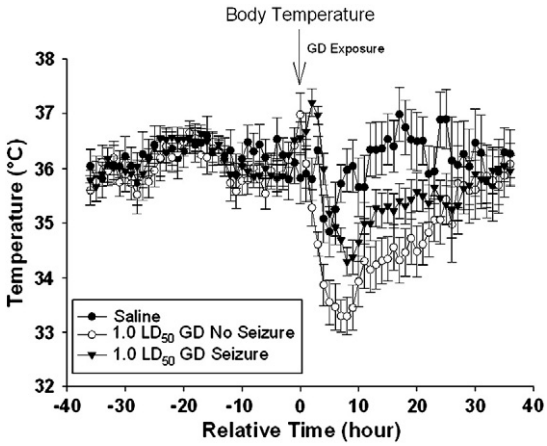

Fig. 1. Body temperature as a function of time relative to GD exposure. GD-exposed rats that did not display seizures (n = 4) had significantly decreased body temperature from 1 to 27 h after GD exposure relative to their baseline. GD-exposed rats that displayed seizures (n = 8) had significantly decreased body temperature from 5 to 24 h after GD exposure relative to their baseline. Saline-treated rats (n = 3) did not have a significant decrease in body temperature in the hours after GD exposure.

Dunnett's post-hoc comparison revealed that the body weights of both GD-exposed groups were significantly less than those of the control group. Tests of simple main effects revealed that the weights of both GD-exposed groups were significantly less than those of the control group throughout the first 15 days post-exposure. Furthermore, the weights of the 1.0 LD_{50} GD no seizure were significantly greater than those of the 1.0 LD_{50} GD seizure group throughout the first 15 days post-exposure.

3.2. Acoustic startle response

There were no differences between groups during the baseline sessions on any measure of startle response. Similarly there were no differences between groups on measures of startle in response to the 60- and 70-dB stimuli either during baseline or post-exposure testing.

3.2.1. Peak startle magnitude (V_{max})

Due to violating the assumption of homogeneity of variance, the V_{max} data for the 100-dB startle eliciting stimuli were $\log_{10}$ transformed prior to the ANOVA. The results of the ANOVA revealed no significant main effect of group [F (2, 19) = 1.03, p = 0.38]; however, the main effect of session [F (5, 93) = 7.24, p < 0.001] and the interaction between group and session [F (10, 93) = 2.28, p < 0.02] were significant. As seen in the upper panel of Fig. 2, the startle magnitude of the 1.0 LD_{50} GD seizure group significantly increased during the post-exposure assessments and was significantly greater than that of the control group during all post-exposure sessions.

As seen in the lower panel of Fig. 2, V_{max} in response to the 120-dB stimuli increased during the post-exposure sessions for those animals exposed to 1.0 LD_{50} GD that experienced seizure activity. The results of the ANOVA revealed that there was not a significant main effect of group [F (2, 19) = 1.06, p = 0.37]; however, the main effect of session was significant [F (5, 93) = 7.52, p < 0.001], and there was a significant interaction between group and session [F (10, 93) = 2.75, p = 0.005]. Tests of simple effects revealed that the 1.0 LD_{50} GD seizure group had higher magnitude startle responses during the post-exposure testing sessions than both the 1.0 LD_{50} GD no seizure and control groups.

3.2.2. Latency to peak startle magnitude (T_{max})

The upper panel of Fig. 3 shows the T_{max} data in response to 100-dB startle stimuli. The results of the ANOVA revealed insignificant main effects of group [F (2, 19) = 0.40, p = 0.67] and session [F (5, 93) = 1.57, p = 0.18]. There was, however, a significant interaction between group and session [F (10, 93) = 2.72, p = 0.006]. As seen in the upper panel of Fig. 3, the 1.0 LD_{50} GD seizure group had decreased T_{max} values, as compared to the control group, during the first and final post-exposure testing sessions. Furthermore, the T_{max} values of the 1.0 LD_{50} GD seizure group were less than those of the 1.0 LD_{50} GD no seizure group during the final post-exposure testing session.

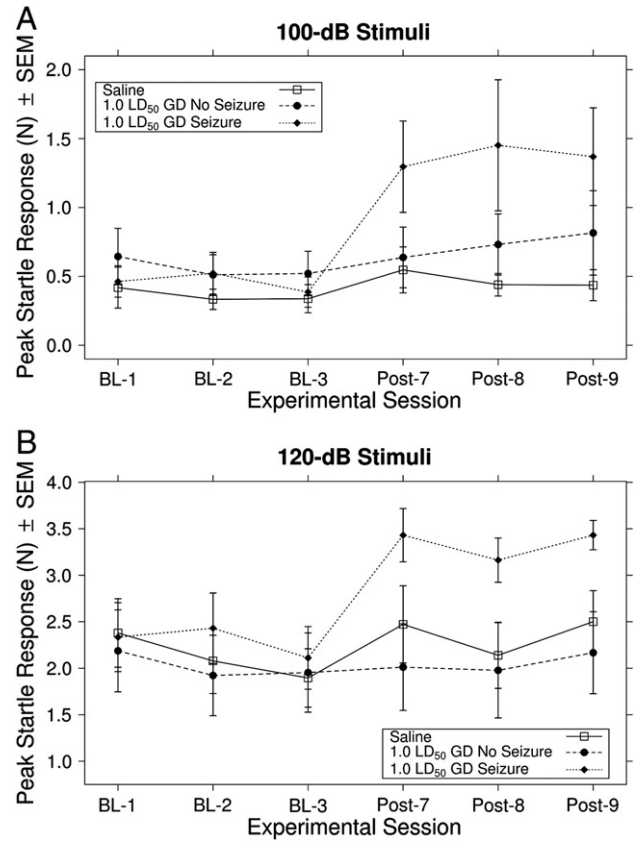

Fig. 2. (A) Startle response magnitude (V_{max}) across sessions for the different groups in response to a 100-dB white noise burst. A single exposure to 1.0 LD_{50} GD that produced seizure activity increased startle magnitude when assessed between days 7 and 9 post-exposure. (B) V_{max} in response to 120-dB stimuli across sessions. A single exposure to 1.0 LD_{50} GD that resulted in seizure (n = 8) activity increased startle response magnitude. Ordinate units are Newtons.

T_{max} data for the 120-dB startle trials were $\log_{10}$ transformed prior to ANOVA due to heterogeneity of variances. The lower panel of Fig. 3 shows T_{max} data for the 120-dB startle trials. The ANOVA revealed no significant main effects of group [F (2, 19) = 1.72, p = 0.21] nor a significant interaction between group and session [F (10, 93) = 1.78, p = 0.08]. There was a significant main effect of session [F (5, 93) = 2.67, p = 0.03], revealing that overall latencies during the first and last post-exposure sessions were lower than those during the final baseline session.

3.2.3. Percent prepulse inhibition (PPI)

PPI data for the 100-dB startle stimulus are presented in the upper panel of Fig. 4. As seen in that figure, there is considerable variability within and between treatment groups. Due to violating the assumption of homogeneity of variance, the data were arcsine transformed prior to ANOVA. The ANOVA confirmed that there were no significant main effects of either group [F (2, 19) = 0.67, p = 0.52] or session [F (5, 93) = 1.1, p = 0.37], nor was there a significant interaction between these factors [F (10, 93) = 0.66, p = 0.76].

The lower panel of Fig. 4 shows the PPI data in response to the 120-dB startle stimulus. The ANOVA revealed a significant main effect

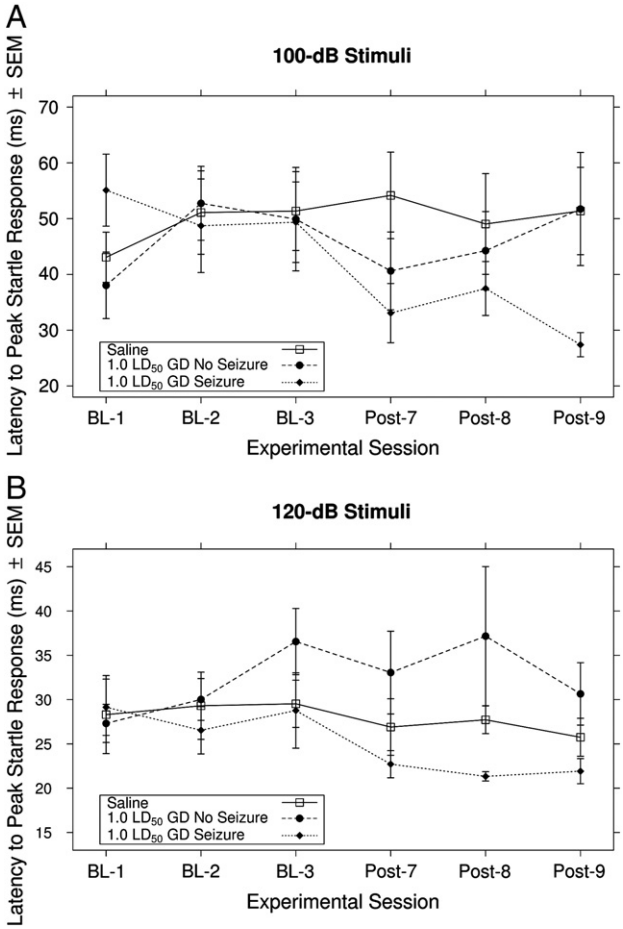

Fig. 3. (A) Latency to peak startle response (T_{max}) across testing sessions in response to 100-dB stimuli. During the first (Post-7) and last (Post-9) post-exposure session, animals exposed to 1.0 LD_{50} GD that had seizure activity had shorter latencies to peak startle than did the control animals. (B) T_{max} in response to 120-dB stimuli. There were no significant differences between groups for T_{max}.

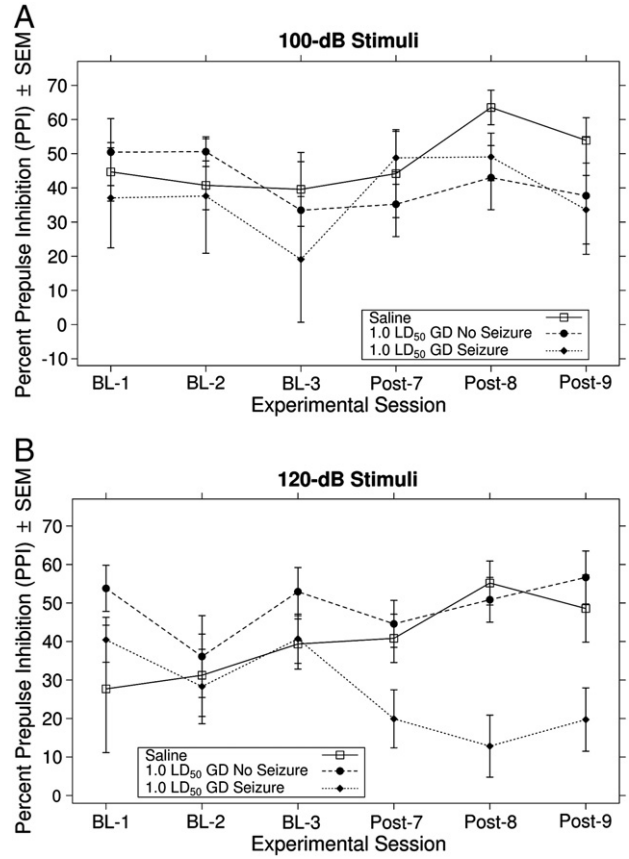

Fig. 4. (A) Percent prepulse inhibition (PPI) in response to 100-dB stimuli with a 70-dB prepulse stimulus. There were no statistically significant differences between groups. (B) PPI in response to 120-dB startle stimulus with 70-dB prepulse stimulus. PPI for the 1.0 LD_{50} GD that experienced seizure activity was significantly less than that of the control group during post-exposure session 1.

of group [F (2, 19) = 7.42, p<0.005]. The post-hoc Dunnett's test revealed that the 1.0 LD_{50} GD seizure group had significantly lower PPI than the control group. There was no significant main effect of session [F (5, 93) = 0.88, p = 0.5], nor was there a significant interaction between group and session [F (10, 93) = 1.83, p = 0.066].

3.3. Autoshaping

There were no significant differences in body weights (data not shown) between groups [F (2, 19) = 1.11, p>0.35], nor was there a significant interaction between group and session [F (28, 266) = 0.52, p = 0.98]. There was a significant main effect of session [F (14, 266) = 117.57, p<0.001], indicating that weights decreased across the 15 sessions of the experiment.

The ANOVA revealed that there were no significant main effects of group [F (2, 19) = 3.49, p = 0.051] on the number of lever presses per session (data not shown). However, there was a significant main effect of sessions [F (14, 266) = 3.32, p<0.001] and a significant interaction between group and session [F (28, 266) = 2.81, p<0.001]. These effects are due primarily to 2 animals in the 1.0 LD_{50} GD no seizure group that began making >15 lever presses per session during the 9th and 10th session, respectively. The data from these 2 animals contributed significantly to the heterogeneity of variances. No data transformations tried (logarithmic, square root, reciprocal) completely eliminated the heterogeneity of variances between the groups. Each dependent variable was aggregated into 3 blocks of 5 sessions each (representing weeks), and a secondary analysis using the nonparametric Kruskal–Wallis was performed. This analysis revealed no significant differences between groups during the first [H = 4.38, df = 2, p = 0.112], second [H = 2.18, df = 2, p>0.35] or third [H = 2.16, df = 2, p>0.33] weeks of testing.

Given that there were no differences between groups in their propensity to display sign-tracking behaviors (i.e., lever presses), it was decided to investigate whether there were differences in goal-tracking behaviors (i.e., head entries into the food trough). Thus, the total number of entries into the food hopper was evaluated (Fig. 5). The ANOVA revealed a significant main effect of group [F (2, 19) = 6.34, p<0.01], and the Dunnett's post-hoc test revealed that the 1.0 LD_{50} GD seizure group made significantly more head entries than the control group. There was also a significant main effect of session [F (14, 266) = 5.12, p<0.001] and a significant group by session interaction [F (28, 266) = 5.26, p<0.001]. The main effect of session revealed that head entries increased across sessions, and the interaction revealed that beginning on the 7th session of training head entries of the 1.0 LD_{50} GD seizure group were greater than those of the control group and the 1.0 LD_{50} GD no seizure group, which were not different from one another.

3.4. Fixed-ratio (FR) transitions

For the analysis of the FR transitions, the data for each subject was averaged within ratio (3 sessions at each ratio requirement) before being subjected to a two-way repeated measures ANOVA (group = between-subjects factor, ratio = within-subjects factor). There were no significant differences in body weight (data not shown) between groups [F (2, 19) = 0.59, p = 0.57], nor was there a significant interaction

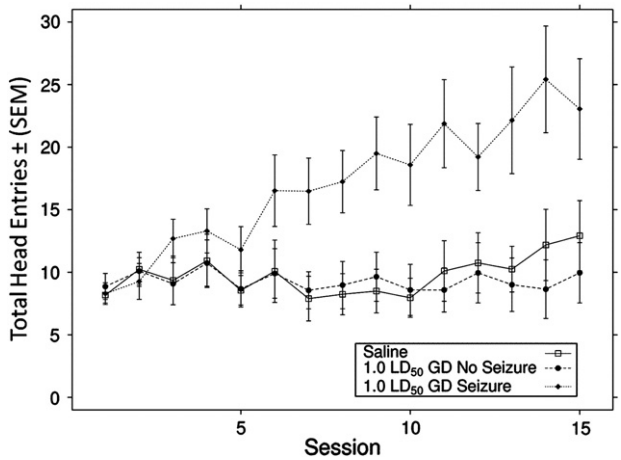

Fig. 5. Shows the total number of head entries per session across the 15 sessions of autoshaping. The animals exposed to 1.0 LD_{50} GD that had seizure activity (n = 8) made significantly more head entries than did the control (n = 8) and 1.0 LD_{50} GD no seizure (n = 6) groups.

between group and ratio requirement [F (8, 76) = 1.08, p>0.38]. There was a significant main effect of ratio [F (4, 76) = 13.25, p<0.001]; body weights during the replication of the FR 5 were less than those during the other ratios.

In terms of behavioral performances, a number of measures were examined: reinforcers earned, overall response rate, within-ratio response rate (running rate), session completion time, total responses, rate of reinforcement, reinforcer collection time, and post-reinforcement pause. Of these measures only reinforcer collection time and post-reinforcement pause did not violate the assumption of homogeneity of variance and were analyzed as the raw values; the remainder were logarithmically transformed prior to the ANOVA. The only measure that revealed a significant main effect of group [F (2, 19) = 8.90, p<0.01] was post-reinforcement pause (PRP; Fig. 6, upper panel). The Dunnett's post-hoc comparison revealed that the control group had longer PRPs than both the 1.0 LD_{50} GD no seizure and 1.0 LD_{50} GD seizure groups. There were also significant main effects of ratio requirement [F (4, 75) = 25.61, p<0.001] and a significant interaction between group and ratio requirement [F (8, 76) = 4.40, p<0.02]. PRPs during the FR 75 were longer than those of any of the other ratio requirements. Similarly, PRPs during the FR 25 were longer than those during the FR 1, FR 5, and FR 5 replication. Tests of simple main effects revealed that both GD-exposed groups had significantly shorter PRPs during the FR 25 and FR 75 phases than did the control group (p-values<0.001).

The time taken to collect the reinforcer (Fig. 6, lower panel) was not significantly different between groups [F (2, 19) = 2.59, p>0.10] or ratio requirements [F (4, 75) = 1.62, p>0.17]; however, there was a significant interaction between group and ratio requirement [F (8, 75) = 2.84, p<0.01]. Tests of simple main effects revealed that the 1.0 LD_{50} GD seizure group took longer to collect reinforcers from the food hopper under ratio requirements greater than FR 1 than did both the control and the 1.0 LD_{50} GD no seizure groups.

An analysis of the number of entries into the food trough (Fig. 7) revealed that the 1.0 LD_{50} GD seizure group made significantly more head entries during the initial evaluation of FR 5 and FR 25 schedules than the control or 1.0 LD_{50} GD no seizure groups. The main effects of group [F (2, 19) = 5.59, p<0.02] and ratio requirement [F (4, 76) = 32.29, p<0.001] were statistically significant; however, the interaction effect was not [F (8, 76) = 1.12, p>0.35]. Tests of simple main effects of group revealed that both the control and 1.0 LD_{50} GD no seizure group made significantly fewer head entries than did the 1.0 LD_{50} GD seizure group. Tests of the simple main effects of ratio requirement revealed that fewer head entries were made during the FR 1 schedule than any other FR schedule requirement. Furthermore, the number of head entries made during the FR 5 and FR 25 schedule requirements was greater than that made during the FR 75 and FR 5 replication, but those were not different from each other.

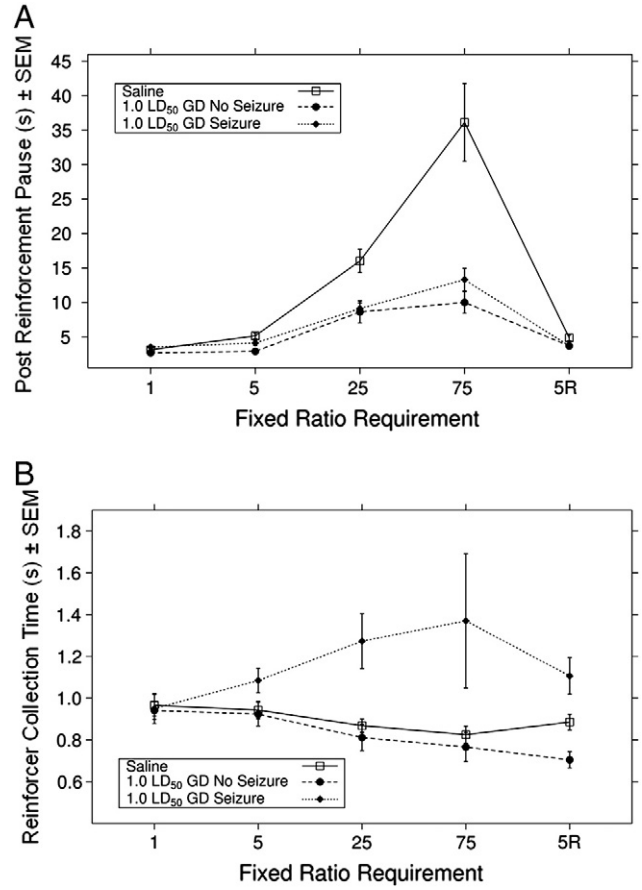

Fig. 6. (A) Post-reinforcement pause as a function of fixed-ratio (FR) schedule requirement. Each point represents the mean of 3 sessions conducted at each FR value. The control group had significantly longer post-reinforcement pauses during the FR 25 and FR 75 schedules than did either group exposed to GD. (B) Time taken to collect the reinforcer from the food hopper. Animals exposed to 1.0 LD_{50} GD that had seizure activity took significantly longer to collect their reinforcers than did the other groups at FR requirements greater than 1. Group sizes were n = 8, 6, and 8 for the saline, 1.0 LD_{50} GD no seizure, and 1.0 LD_{50} GD seizure groups, respectively.

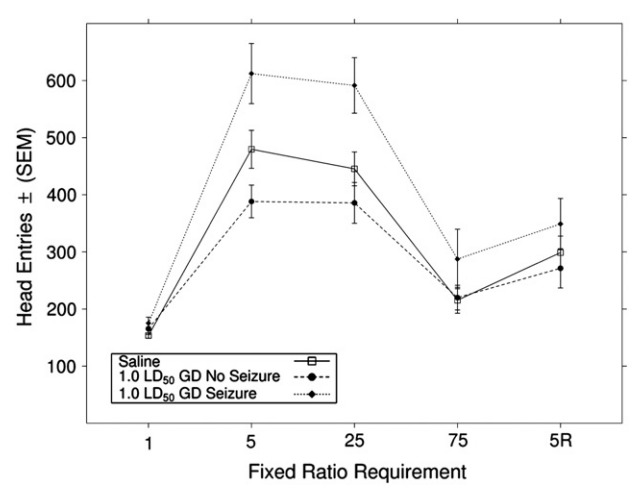

Fig. 7. Group mean head entries per session ± SEM as a function of FR schedule value. Animals previously exposed to GD that experienced seizure activity made more head entries than did the saline animals. This difference was most prominent at FR schedule requirements of FR 5 and FR 25.

For the remaining measures of behavioral performance during the FR transitions, there were no significant main effects of group or significant interactions between group and ratio. For those measures, there were significant main effects of ratio, generally indicating that performance varied as a function of ratio requirement (data not shown).

3.5. Neuropathology

Rats exposed to GD that experienced seizure activity had extensive fiber degeneration in the piriform cortex, thalamus, fiber tracts (cingulum, external capsule, internal capsule) and to a lesser extent the amygdala and hippocampus (Fig. 8A). Control animals and non-seizing animals exposed to GD had equally negligible levels of neuropathology. As seen in Fig. 8B, the thalamus and fiber tracts had the most extensive pathology, whereas the piriform cortex had moderate pathology. There was minimal pathology in amygdala and hippocampus at the time of tissue collection (approximately 80 days post-GD exposure).

4. Discussion

The present investigation examined the effects of GD-induced seizure activity using a series of behavioral assessments. The results of the present investigation revealed that GD-exposed rats that experienced seizure activity had elevated startle responses compared to controls. There were no detectable differences due to GD exposure/seizure activity on the acquisition of lever pressing during autoshaping. There were, however, large differences in goal-tracking (i.e., food trough-directed) behaviors. The 1.0 LD_{50} GD seizure animals engaged in more goal-tracking behaviors than either the control animals or the 1.0 LD_{50} GD no seizure animals. There were also differences between groups on measures of post-reinforcement pause and reinforcer collection time during the development of FR schedule appropriate behavior. Furthermore, the 1.0 LD_{50} GD seizure animals engaged in perseverative food trough behaviors at moderate FR schedule values. Only animals that experienced seizure activity as a result of GD exposure had appreciable neuropathology. The fiber tracts, piriform cortex, and thalamus were the brain regions predominantly affected by GD-induced seizure activity. Minimal damage was observed in the amygdala and hippocampus, which is consistent with previous results from our laboratory (Moffett et al., 2011). However, it is quite possible that damage to these areas was underestimated since the silver-staining procedure primarily identifies degenerating axons rather than neurons (Switzer, 2000).

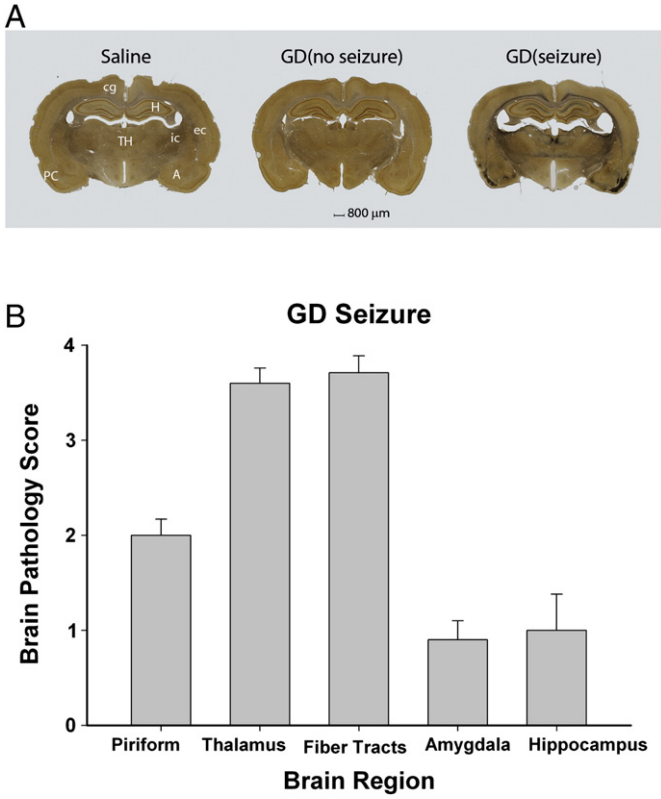

Fig. 8. A. Silver-stained coronal sections (approximately −3.00 mm from bregma) showing extensive fiber degeneration in piriform cortex (PC), thalamus (TH), fiber tracts (cingulum (cg), external capsule (ec), internal capsule (ic)), and to a lesser extent amygdala (A) and hippocampus (H) of rats exposed to 1.0 LD_{50} GD that displayed prolonged seizure activity. Left (saline); middle (GD no seizure); right (GD seizure). B. Bar graph shows rank scoring of damage of rats exposed to GD that displayed seizures. No damage was observed in saline control or in GD-exposed rats that did not display seizures (data not shown).

Although other laboratories have studied the effects of GD on ASR, the majority of studies have been performed using guinea pigs. Philippens et al. (2000, 2005) observed increased ASR in guinea pigs 2 h after exposure to 2.0 LD_{50} (49 μg/kg, sc) GD, but not one week after exposure. In contrast, Haggerty et al. (1986) observed reduced ASR amplitude and increased ASR latency in rats 2 h after exposure to 0.8 LD_{50} (150 μg/kg, im) GD; longer term effects on ASR were not reported. In the current study, we observed increased ASR amplitude and reduced latency to peak startle one week after exposure to 1.0 LD_{50} GD, but only in rats that displayed seizures and neuropathological damage. In addition, we observed significantly less PPI in rats exposed to 1.0 LD_{50} GD that displayed seizures. The interpretation of the decreased PPI is difficult due to the increased startle magnitude on pulse-only trials (Swerdlow et al., 2000, 2001). However, a deficit in PPI has been reported in other seizuregenic animal models (Ma et al., 2004; Ma and Leung, 2010). Moreover, Wolf et al. (2010) observed a negative correlation between PPI levels and the extent of ibotenic acid-induced lesions in the ventral thalamus of rats. Lesions in the basolateral amygdala have also been shown to decrease PPI in rats (Wan and Swerdlow, 1997), and damage to both of these regions was observed in GD-exposed rats that experienced seizure activity.

While there were no detectable differences on the acquisition of lever pressing during autoshaping, the current investigation did reveal that GD-induced seizure activity resulted in perseverative responding (i.e., food trough-directed behaviors) during both autoshaping and the development of FR schedule performance. The development of perseverative behaviors is consistent with previous reports investigating the effects of GD on the development of operant performances (McDonough et al., 1986; Modrow and Jaax, 1989). McDonough et al. (1986) showed that rats exposed to 1.0 LD_{50} (110 μg/kg, sc) GD had difficulty acquiring DRL schedule performance. In that study, the GD-exposed animals' overall levels of lever pressing were not different from that of control animals; however, GD-exposed animals earned fewer reinforcers per session. The derived measure, response efficiency (lever presses/reinforcers), revealed that GD-exposed animals were responding in a less efficient manner than controls. Furthermore, an analysis of IRT (inter-response time) distributions revealed that GD-exposed animals persistently responded with predominately short IRTs. In a later study, Modrow and Jaax (1989) examined the acquisition of cued delayed-alternation performance in rats that had been exposed to GD doses less than 1.0 LD_{50} (75–95 μg/kg, sc). The primary finding of that study was a dose-related increase in the number of sessions required to meet the acquisition criterion (<25% responses on incorrect lever for 3 consecutive sessions). Unlike the McDonough paper, the Modrow paper did not provide data on the number or nature of the errors the animals made. However, given the requirements of a cued delayed-alternation task, it is reasonable to infer that the animals were making perseverative errors either within or across trials that prevented them from reaching the acquisition criterion. Furthermore, Cohen and Poplawsky (1982) showed that rats with septal lesions that extend into the hippocampus, an

area of the brain damaged (albeit minimally) by GD-induced seizure activity, have an increased tendency to perseverate on an incorrect lever, thus increasing the number of errors committed during an FR schedule. Similarly, Kimble and Kimble (1965) showed that rats with bilateral hippocampal lesions made significantly more errors during a reversal task on the Y-maze due to their inability to give up the initially learned position. Interestingly, Gralewicz et al. (2000) showed a positive correlation between the number of perseveration errors committed during a radial arm maze task with the total duration of spike-and-wave discharge activity in rats. Additionally, Arkhipov et al. (2008) observed an increase in perseverative responding during the extinction of a food-procuring task in rats injected with a subconvulsive dose of kainic acid. Thus, it may come as no surprise that 11% of patients with temporal lobe epilepsy have also diagnosed with an obsessive compulsive disorder (de Oliveira et al., 2010).

Using telemetry to continuously record body temperature, we observed that GD exposure resulted in the transient reduction of body temperature in both rats that displayed seizures and in non-seizing rats. These findings of nerve agent-induced transient hypothermia are in agreement with our previous findings of GD inhibition of body temperature in rats exposed to 1.0 LD_{50} GD with standard therapy (de Araujo Furtado et al., 2009) and in rats exposed repeatedly (1/day × 3 days) to 0.4–0.8 LD_{50} VX (Lumley et al., 2006). In addition, Meeter and Wolthuis (1968) observed a dose-dependent decrease in body temperature with GD-exposed rats; body temperature fell 4–6 °C in the first 3 h after exposure and subsequently returned to baseline by 24 h. Clement (1991, 1993) also observed transient hypothermia in mice exposed to GD, with temperature returning to pre-exposure levels within 24 h of exposure, and observed that atropine sulfate prevented inhibition of body temperature by sarin, suggesting that this effect is mediated by muscarinic receptors.

In summary, the present investigation found evidence of both short-term dysfunction following nerve agent-induced seizures and long-term behavioral alterations. The present data indicate that ASR is elevated in animals that experience seizure activity resulting from GD exposure. Furthermore, those animals also had decreased PPI. Provided these findings are replicated, ASR may be a useful screening tool for evaluating putative neuroprotective drugs. Autoshaping failed to reveal differences in the acquisition of lever pressing based upon exposure history/seizure status. However, we were able to determine that perseverative goal-tracking (food trough-directed behavior) is increased in GD-exposed animals that experience seizure activity. Future directions indicate that manipulations of the temporal parameters (ITI duration, CS duration, and trace duration) of the autoshaping procedure may be beneficial in engendering levels of lever pressing that will differentiate between controls and GD-exposed/seizure animals. The development of FR schedule performance revealed that animals that had experienced GD-induced seizure activity engaged in similar perseverative behaviors. Future assessments of the acquisition of operant behavior may include procedures that require the animal to respond based on the passage of time as these may be more sensitive to the underlying neuropathology of nerve agent-induced seizures (Bizot, 1998; McDonough et al., 1986; Reilly and Good, 1989) than ratio schedules of reinforcement. The current experiments provide valuable data on the behavioral consequences of GD-induced seizure activity as well as future directions to pursue in the development of this behavioral model for evaluating the efficacy of putative neuroprotective compounds.

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The study sponsors had no involvement in the study design, analysis, interpretation of data, manuscript preparation or in the decision to submit the manuscript for publication. We have no conflicting interests to declare.

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